

Enzymatic resistance to the lipopeptide surfactin as identified through imaging mass spectrometry of bacterial competition

B. Christopher Hoefler^a, Karl V. Gorzelnik^a, Jane Y. Yang^b, Nathan Hendricks^b, Pieter C. Dorrestein^b, and Paul D. Straight^{a,1}

^aDepartment of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843; and ^bSkaggs School of Pharmacy and Pharmaceutical Sciences, University of California at San Diego, La Jolla, CA 92093

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Many species of bacteria secrete natural products that inhibit the growth or development of competing species. In turn, competitors may develop or acquire resistance to antagonistic molecules. Few studies have investigated the interplay of these countervailing forces in direct competition between two species. We have used an imaging mass spectrometry (IMS) approach to track metabolites exchanged between *Bacillus subtilis* and *Streptomyces* sp. Mg1 cultured together. Surfactin is a cyclic lipopeptide produced by *B. subtilis* that inhibits the formation of aerial hyphae by streptomycetes. IMS analysis exposed an addition of 18 mass units to surfactin in the agar proximal to *Streptomyces* sp. Mg1 but not other streptomycetes tested. The spatially resolved change in the mass of surfactin indicated hydrolysis of the molecule. We observed that the aerial growth of *Streptomyces* sp. Mg1 was resistant to inhibition by surfactin, which suggests that hydrolysis was a mechanism of resistance. To identify possible enzymes from *Streptomyces* sp. Mg1 with surfactin hydrolase activity, we isolated secreted proteins and identified candidates by mass spectrometry. We purified one candidate enzyme that hydrolyzed surfactin in vitro. We tested the role of this enzyme in surfactin resistance by deleting the corresponding gene from the *S. Mg1* genome. We observed that aerial growth by the $\Delta sfhA$ mutant strain was now sensitive to surfactin. Our results identify an enzyme that hydrolyzes surfactin and confers resistance to aerial growth inhibition, which demonstrates the effective use of an IMS approach to track natural product modifications during interspecies competition.

antibiotic resistance | microbial ecology | secondary metabolism | surfactant

Competition among bacterial species involves the exchange of natural product metabolites, including antibiotics, signals, and toxins (1–6). Natural products benefit producing bacteria through signaling and inhibitory functions toward competitor bacteria (7–9). However, many of these metabolites provide a powerful selection for resistance to emerge within bacterial communities. Recent surveys of microbial communities have demonstrated the widespread nature of antibiotic resistance in the microbial world (10–12). The prevalence of antibiotic producing bacteria in the environment accords with the diverse resistance mechanisms that detect and defuse a range of bioactive natural products (reviewed in ref. 13). One of the hallmark forms of antibiotic resistance is the enzymatic degradation or modification of specific xenobiotic metabolites (14). For example, β -lactamases hydrolyze penicillins, which block their cell wall inhibitory activity and consequent lysis of the exposed cells (reviewed in ref. 15). Enzymatic mechanisms of resistance are not limited to antibiotics. Degradative enzymes that impact competitive interactions also include those that degrade metabolites with signaling functions. For instance, quorum-quenching activities, such as homoserine lactonases, degrade the quorum-signaling compounds that regulate cell-density-dependent functions in competing species (16–18). Enzymes that degrade or modify natural products provide protection by blocking the antagonistic and competitive functions. However, beyond antibiotic

resistance, relatively little is known about enzymatic transformations of secreted metabolites that occur during competitive interactions between species of bacteria.

This report focuses on an antagonistic function of the natural product surfactin during bacterial competition between two soil organisms and a mechanism of resistance toward surfactin. Surfactin is a cyclic lipodepsipeptide that is secreted by species of *Bacillus* and disrupts the growth and development of other organisms (19–22). The microbial functions described for surfactin encompass its powerful surfactant activity, antibiotic and antiviral activities, and a recently described paracrine signaling function during biofilm development by *B. subtilis* (23–29). In prior studies of competitive interactions between *Streptomyces coelicolor* and *B. subtilis*, surfactin was found to inhibit streptomycete development of aerial hyphae and spores (30, 31). Despite multiple examples of its antagonistic effects, mechanisms of microbial resistance to surfactin have yet to be described. Instability of surfactin in soils has been reported, suggesting degradation by microorganisms in the environment, but mechanisms for the degradation of surfactin are unknown (32).

Surfactin acts primarily on cellular membranes to disrupt membrane integrity (33). The micelle-forming properties of surfactin, which complement the membrane active properties of the molecule, may complicate the identification of a resistance mechanism. The cyclized peptide moiety of surfactin folds into a “horse saddle” structure that contributes to the exceptional surfactant properties and stability of the molecule (34). The surface activity and stability are in part due to incorporation of L- and D-amino acids, which facilitate folding of the peptide to form an amphipathic headgroup. The complete surfactin molecule also includes a hydrophobic acyl chain, which varies in length, with C₁₃, C₁₄, and C₁₅ forms predominant (34, 35). Complete surfactin monomers assemble into micelles at a low critical micellar concentration (CMC), enhancing the surfactant properties of the molecules (36). The overall stability of surfactin most likely arises from the combination of the folded peptide structure, the incorporation of D-amino acids, and the self-association of the monomers. Evidence for limited proteolytic susceptibility of surfactin was discovered in an in vitro study of purified V8 endoprotease from *Staphylococcus aureus* (37). Surfactin peptide cleavage was minimal in these experiments, degrading ~14% of the total metabolite pool, despite extensive incubation with the enzyme. Thus, the identity of enzymes that efficiently degrade surfactin remains uncertain.

Here, we report the identification of a mechanism of surfactin resistance from a soil actinomycete, *Streptomyces* sp. Mg1

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¹To whom correspondence should be addressed. E-mail: paul_straight@tamu.edu.

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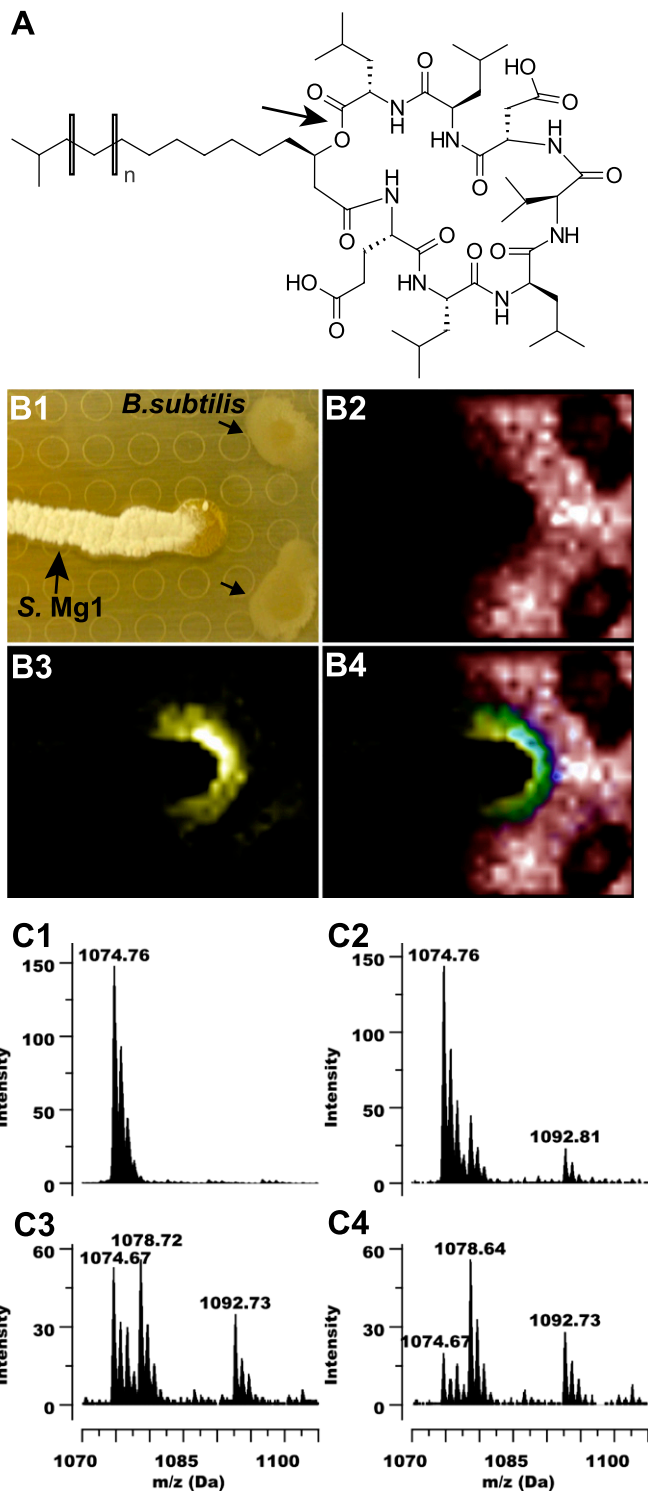


Fig. 1. IMS of surfactin from *B. subtilis* 3610 with *Streptomyces* sp. Mg1. (A) The structure of surfactin with the variable length acyl chain indicated in brackets ($\|_n$), and the bond forming the lactone marked with an arrow. (B1) Image of *B. subtilis* and *S. Mg1* cultured for IMS (*Materials and Methods*). *S. Mg1* (vertical arrow) was inoculated as a streak of spores. *B. subtilis* (small arrows, right) was inoculated in two spots to the right of *S. Mg1*. The images (B2–B4) depict ions for C_{13} -surfactin (m/z 1047, red; B2) and hydrolyzed (+18 m/z) C_{13} -surfactin (m/z 1065, yellow; B3). An overlay of B1 and B2 illustrates the relative spatial distribution of ions (B4), revealing surfactin hydrolysis in proximity to the *S. Mg1*. (C) Single spectra from the imaging data of a *B. subtilis* 3610 and *S. Mg1*. An increase in surfactin +18 m/z ion intensity occurs with a corresponding reduction in the intact surfactin ion intensity.

(*S. Mg1*), in a competitive interaction with *B. subtilis*. To follow the fates of surfactin and other natural products in competition with *S. Mg1*, a MALDI-TOF-based imaging mass spectrometry (IMS) approach was used (31, 38, 39). IMS provided a method to track in situ the secretion and modification of surfactin during interspecies interactions. The IMS data suggested that *S. Mg1* hydrolyzes surfactin. Specifically, we observed a spatially localized +18 *m/z* mass change for surfactin as it diffused from colonies of *B. subtilis* toward colonies of competing *S. Mg1*. Several species of *Streptomyces* were tested for sensitivity to surfactin-induced balding. *S. Mg1* was found to be the only species of those tested that could form aerial hyphae in the presence of surfactin. The detection of surfactin hydrolysis during interspecies competition led to efficient identification and purification of the hydrolyzing enzyme produced by *S. Mg1*. We reasoned that this enzyme could provide a surfactin resistance mechanism for *S. Mg1*, which was confirmed by deletion of the gene encoding the surfactin hydrolase.

Results

Imaging Mass Spectrometry Reveals Surfactin Degradation. In a survey of streptomycetes cultured with *B. subtilis* NCIB3610, we observed that aerial growth and spore development were disrupted to varying degrees for several species of *Streptomyces* (Fig. S1). Based on our previous observation that surfactin inhibits aerial development of *S. coelicolor*, which we describe as a balding effect, we hypothesized a role for surfactin in these competitive interactions (Fig. 1A) (30, 31). However, the relative differences in growth, development, and metabolism of the two organisms in coculture obscure the functions of individual metabolites. As an approach to monitoring natural products, including surfactin, during competitive interactions between *B. subtilis* and streptomycetes, we used MALDI-imaging mass spectrometry (IMS) (31, 40). *B. subtilis* and *Streptomyces* spp. are commonly isolated from soils where they compete for resources when they are actively growing. We focused on one species in particular, *Streptomyces* sp. Mg1 (*S.* Mg1), because we isolated the strain in parallel with strains of *B. subtilis* from a single sample of soil in which the species may naturally compete (41).

We used IMS to monitor the production and fate of natural product metabolites in the competitive interaction between *B. subtilis* NCIB3610 and *S. Mg1* (31, 40). Application of IMS enabled spatial tracking of metabolites in the mass range that we surveyed, 500–3000 *m/z*. For surfactin, multiple ions are detected as a result of its variable-length acyl chain (Fig. 1A). Individual surfactin ions are separated by 14 mass units: *m/z* 1047 [*C*13 + *K*]⁺, *m/z* 1061 [*C*14 + *K*]⁺, and *m/z* 1075 [*C*15 + *K*]⁺. This typical pattern of surfactin ions was detected in the space between the *B. subtilis* colonies and *S. Mg1* (Fig. 1B2). Near the *S. Mg1* colony, new ions could be identified from the spectra in the same mass range as the surfactins. Curiously, we noted that with increasing proximity to *S. Mg1*, the newly visible peaks in the IMS spectra corresponded to each surfactin ion +18 *m/z* (Fig. 1B3 and C). Progression through the sequence of images of Fig. 1 illustrates the spatial distribution of the new ions. Individual mass spectra from selected positions in the coculture show an increase in intensity of the new ions near *S. Mg1* (surfactin +18 = *m/z* 1093) concomitant with a decrease in the intensity of the parent surfactin ion (*m/z* 1075). This observed pattern suggests the +18 *m/z* ions are surfactins undergoing hydrolysis by an activity secreted from *S. Mg1*. To confirm the +18 *m/z* ions were related to the surfactin ions, and not unique metabolites secreted by *S. Mg1*, we fragmented the ions by using tandem MS/MS. The resulting patterns of *b*- and *y*-ion fragmentation for the +18 *m/z* ions were

The spectral window is limited to the range m/z 1070–1105 to highlight representative C₁₅-surfactin [M+K]⁺ (m/z 1074.76) and hydrolyzed surfactin ions [M+K+H₂O]⁺ (m/z 1092.81) in succession from near the *Bacillus* colony (C1), at the midpoint between the colonies (C2), and near the *S. Mg1* colony (C3 and C4). Note: m/z 1078.72 is the hydrolyzed [M+K+H₂O]⁺ ion of C₁₄-surfactin (m/z 1060.75).

Table 2. Surfactin hydrolase specificity for surfactin and plipastatin compared with other potential lipopeptide and macrocyclic substrates

Compound	Hydrolysis*
Surfactin	+
Plipastatin	±
Iturin A	—
Daptomycin	—
A54145D	—
Nystatin	—
Amphotomycin	—
Amphotericin B	—
Erythromycin	—
CDA [†]	—

The purified hydrolase was incubated with the metabolites listed for 30 min. +, ~95% hydrolysis; ±, some hydrolysis; —, no hydrolysis detected under conditions used.

*See Fig. S5 for corresponding mass spectra.

[†]Calcium-dependent antibiotic.

1.10×10^{-9}) and belongs to the esterase-lipase superfamily based on the conserved catalytic core. Despite the presence of genes encoding similar hydrolases, we did not detect surfactin hydrolysis activity from other *Streptomyces* species tested. The *S. Mg1* enzyme may have some intriguing structural differences that confer substrate specificity for surfactin but also show diminished hydrolysis of plipastatin and possibly other lipopeptides. Together with our experimental results, the primary sequence information for the enzyme supports its observed function as a lipopeptide hydrolase variant of a larger family of secreted hydrolases.

Surfactin belongs to the lipodepsipeptide class of natural products, which includes antibiotics such as daptomycin and ADEPs (46, 47). Although the surfactin hydrolase had activity against the lipopeptide plipastatin in addition to surfactin, other compounds tested were not substrates for the enzyme. The ability of the enzyme to hydrolyze two structurally different substrates suggests that one or a few random mutations in the surfactin hydrolase gene may produce an enzyme permissive for hydrolysis of daptomycin or other lipopeptides. Studies on enzyme promiscuity toward substrates such as plipastatin and daptomycin, as well as structural characterization of the substrate-binding domain, will be of interest. This report demonstrates that bacterial competition and IMS is a useful tool for discovery of activities that degrade many types of natural products. In particular, the imaging strategy described is a unique method for discovering and understanding antibiotic resistance and could be applied to a range of microbial communities and interactions.

Materials and Methods

Bacterial Strains and Media. The strains used for this study were *B. subtilis* 3610 (PDS0066), *S. Mg1* wild type (PSK0558), $\Delta sfhA$ (PDS0366), and $\Delta sfhA$, *sfhA* ApR (PDS0424). Other *B. subtilis* and *Streptomyces* spp. are as listed in *SI Materials and Methods*. Spore suspensions of *Streptomyces* spp. were prepared by using standard procedures (48) and used for the described plating experiments. Bacto media reagents were used unless otherwise indicated. Media for *Streptomyces* growth [Glucose, Yeast-Extract, Malt-Extract (GYM) and Malt-extract Yeast-extract Maltose (MYM)] and coculture conditions are described in *SI Materials and Methods*. Surfactin, amino acids, thiamine-HCl, Mops, and Universal Matrix were purchased from Sigma-Aldrich along with other chemical reagents, unless otherwise noted. All solvents used were HPLC grade. The *E. coli* WM3780 strain was used as the donor strain for conjugation with *S. Mg1* (49). *E. coli* XL10-Gold and XL1-Blue strains (Stratagene) were used for cloning, and the *E. coli* Rosetta (DE3) strain (Novagen) was used for protein overexpression. For long-term storage, bacterial strains were preserved cryogenically at -80°C in water (*Streptomyces* spp. spores) or 20% (vol/vol) glycerol (*B. subtilis* and *E. coli*). The primers used in this study are listed in Table S4.

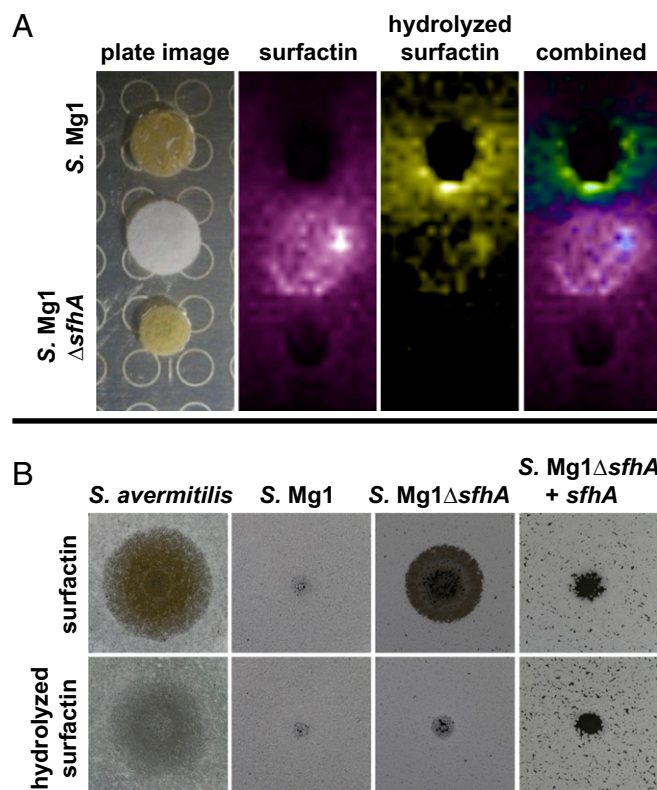


Fig. 4. The $\Delta sfhA$ mutant strain does not hydrolyze surfactin and is sensitive to surfactin-induced balding. (A) Purified surfactin (70 μg) was placed on a filter between the wild-type (Upper) and $\Delta sfhA$ (Lower) strains. The IMS depicts hydrolysis of the *m/z* 1074 ion of surfactin (lavender) to the *m/z* 1092 (+18 *m/z*) form (yellow) by the wild-type strain but not the $\Delta sfhA$ strain. (B) Untreated surfactin (Upper) or hydrolyzed surfactin (Lower) (~100 μg) was applied to lawns of *S. avermitilis*, wild-type *S. Mg1*, the $\Delta sfhA$, and the complemented ($\Delta sfhA$ + *sfhA*) strains. After the onset of aerial development, "bald" patches due to surfactin are on the plates. *S. avermitilis* is highly sensitive to cyclized surfactin but not to enzymatically hydrolyzed surfactin. Wild-type *S. Mg1* produces aerial hyphae in the presence of both cyclized and hydrolyzed surfactin. Aerial hyphae are blocked by surfactin, which is seen as a bald patch on the $\Delta sfhA$ mutant strain of *S. Mg1*. The genetically complemented mutant ($\Delta sfhA$ + *sfhA*) grows aerial hyphae similar to wild type when treated with surfactin. When hydrolyzed by the purified enzyme, surfactin does not block aerial hyphae.

Cultures of *Streptomyces* spp. with *B. subtilis* or Purified Surfactin. Coculture experiments were generally carried out as previously described (30) (*SI Materials and Methods*). The spore density for plating was varied between 10^4 and 10^6 spores per 100-mm plate to obtain consistent and even aerial development across the plate. Buffered GYM was the preferred media for coculture, but supplemented MYM was used for *S. pristinaespiralis*. Mineral supplemented, buffered MYM was used for surfactin tests, except in the case of *S. aizunensis* where supplemented, buffered GYM was used. Purified surfactin was dissolved in DMSO at a concentration of 20 mg/mL. Hydrolyzed surfactin was prepared enzymatically, as described for the NMR analysis (*SI Materials and Methods*), and redissolved after desalting to a concentration of 20 mg/mL in DMSO. Aliquots (5 μL) of surfactin, hydrolyzed surfactin, or DMSO as a negative control were added to the center of each plate. The plates were incubated at 30°C as described for cocultures until aerial hyphae were observed.

MALDI IMS. The format for coculture of *B. subtilis* with *S. Mg1* (*SI Materials and Methods*) was altered slightly to be more suitable for the MALDI imaging experiments. A spore suspension was inoculated in a dense format as either a line across the plate, or in evenly spaced 6- μL drops across the plate (Fig. S3). *B. subtilis* was inoculated (2 μL of an LB overnight culture) adjacent to the *S. Mg1*. After incubation on thin agar plates (*SI Materials and Methods*), the agar layer was separated from the plates and used for MALDI imaging. Cultures were prepared for MALDI imaging by transferring the thin agar sections to a Bruker MSP 96 anchor plate. Universal Matrix was

deposited evenly over the agar by using a 53- μ m test sieve. The agar with matrix was then dried in a 40 °C oven for 1–3 h until desiccated. The sample was dusted with a nitrogen stream and then inserted into a Microflex Bruker Daltonics mass spectrometer for data collection. The data were filtered manually by selecting ions of interest from the average spectrum or from individual spectra and is presented without normalization.

For IMS of wild type and Δ sfhA S. Mg1 activity, 1 μ L of each spore stock (10^9 spores per mL; PSK0558 and PDS0366) were spotted 1.5 cm apart on a 10-mL GYM (2% agar) plate. Once dry, 3 μ L of 23 mg/mL surfactin (69 μ g) was placed on a filter disk between the inoculated spots. The plate was sealed with parafilm and incubated upside down at 30 °C for 48 h. Then, the sample was cut out of the plate and transferred to the MALDI target plate. The filter disk was removed, Universal Matrix was applied through a 53- μ m sieve, and the sample was dehydrated at 37 °C. After the excess matrix was removed, IMS was performed in linear mode by using a Bruker Autoflex Speed at 600- μ m resolution after calibrating to the Peptide Calibration Standard (Bruker 20619). Data were acquired from 500 to 1500 Da at 952 measurement points. The images were acquired by using a raster program as described (31). Extracted ion images were prepared by using TissueView 1.1 (AB SCIEX) and colored in Adobe Photoshop CS3.

Enzyme Activity Assays. Surfactin (50 μ M, mixture of lipopeptides) and other antibiotics assayed (50 μ M each) were reacted with purified hydrolase (320

ng) at ambient temperature in 100- μ L assay buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, and 1 mM DTT). Reactions were quenched with 500 μ L of acetone. Surfactin samples were quenched at time points of 0, 10, 20, 40, 60, and 120 min. A saturated solution of 20–30 mg/mL Universal Matrix dissolved in 1:1 acetonitrile:water with 0.2% TFA (1.0 μ L) was cospotted on a MALDI plate with the quenched reaction mixture (1.0 μ L), and MS1 spectra were collected on a Shimadzu Axima-CFR MALDI-TOF mass spectrometer. The extent of surfactin hydrolysis was determined (semi-quantitatively) by comparing the ratios of intact surfactin ion ($[M+Na]^+$) to hydrolyzed surfactin ion ($[M+Na+H_2O]^+$) for representative ions at the different time points.

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